EXPERIMENTS ON THE VARIABILITY OF THE FERMENTATIVE REACTION OF BACTERIA, ESPECIALLY THE STREPTOCOCCI *

WILLIAM C. THRO

(From the Department of Clinical Pathology of the Cornell Medical College, New York City)

Within the past few years certain chemical substances, especially the carbohydrates, have been used in the separation of species of bacteria. It is in the separation of the members of the typhoid-colon group and the Gram-negative cocci[1] that these substances have been of especial service. These chemicals have also been used by a number of investigators in separating the streptococci into groups. Further, some of them have considered that the fermentation tests might be of practical value in determining the types of streptococci concerned in water pollution.

After trying only a few strains of streptococci on some of these fermentable substances, I was impressed by the great variability of the results, and on reading the literature of the subject I found that previous investigators of the same group of bacteria did not agree. It seemed to me that it would be profitable, after establishing the fact that there was variability, to try to discover the causes.

By perusal of extracts from articles of my predecessors, one can readily see that their results are not in entire accord, and that some of them are very skeptical as to the value of the carbohydrates in separating streptococci. Out of 300 streptococci from saliva, Gordon[2] found that there were no less than 48 types, all of which fermented saccharose, and none fermented mannite. He gives this conclusion after trying out strains pathogenic for man. "These and the other results recorded in the table indicate that there is considerable diversity amongst streptococci occurring in sepsis."

Houston[3] examined 300 strains from feces using eight of Gordon's tests, and found forty types.

Andrewes and Horder[4] believe that if Gordon had used twenty tests instead of nine that instead of forty he would have found one hundred or more varieties amongst them.

Buerger[5] tested thirty-four strains of streptococci using sugar-free broth or beef-serum and water and found six varieties with the carbohydrates among only thirty-three strains.

* Received for publication November 15, 1913.

1. *Jour. Med. Research,* 1909, 20, p. 369.
2. *Lancet,* 1905, 2, p. 1400.
3. *Suppl. Ann. Rept.,* 1904, p. 326.
4. *Lancet,* 1902, 2, p. 708, 775, 852.
5. *Jour. Exper. Med.,* 1907, 19, p. 428.

Winslow and Palmer[6] investigated 302 cultures from feces. Comparing their results with those of Andrewes and Horder they state, "The various investigations are concordant with the exception that rhamnose-fermenters in both human and bovine feces were less frequent than in those of their predecessors."

E. W. Ainley Walker says: "The reactions for the streptococci exhibit an extraordinary degree of variability if observed over extended periods of time, or after changes in their environment likely to encourage the appearance of variations. It follows that the streptococci cannot be classified into varieties and sub-varieties on the results of their reactions in sugar-containing media."

Libman and Celler[8] found that inulin as a substance to differentiate between streptococci and pneumococci is not constant, as out of sixty-nine streptococci, two fermented inulin, and of the nineteen pneumococci, two did not.

Beattie and Yates[9] state: "In our hands Gordon's tests have proved quite unreliable in differentiating strains of streptococci."

Gordon[10] in his reply to Andrewes and Horder's criticism states that Twort has shown that the bacillus typhosis can be coaxed into fermenting lactose. He says, "I will not admit that streptococci are extraordinarily variable in their reactions."

Bergey[11] concludes that the study of the streptococci, for the purpose of differentiating cultures, derived from different sources, has strongly emphasized the unsatisfactory nature of the method of differentiation through carbohydrate fermentation.

Broadhurst[12] used six fermentable substances and one hundred strains of streptococci (from milk). They fell into twenty groups.

Stowell, Hilliard and Schlesinger[13] found that a comparison of their work with that of previous workers does not show any great uniformity in results. In regard to the stability of the fermentation tests they conclude that reinoculation after a week or longer usually confirmed the first findings.

In my work the strains of streptococci isolated from different sources were tried on the following chemicals: dextrin, arabinose, mannite, salicin, raffinose, lactose, saccharose and inulin. Dextrose, galactose, dulcite, maltose and levulose were not used to any great extent as they are not of much use in differentiating streptococci. Agar, containing sugar-free broth from fresh meat and made neutral to Kubel Tiebmann's litmus, was used. The carbohydrates (Merck's or Kahlbaum's) were sterilized separately for only fifteen minutes in the Arnold sterilizer and added to the melted agar, which was then incubated at 37 C. for two days. The contaminated tubes were then discarded. All the strains were tried in blood agar and by this means were separated into three groups, that is, those producing green pigment, those producing hemolysis, and those producing pneumococci.

6. *Jour. Infect. Dis.*, 1910, 7, p. 1.
7. *Jour. Path. and Bacteriol.*, 1911, 15, p. 124.
8. *Am. Jour. Med. Sc.*, 1910, p. 140, 516, 527.
9. *Jour. Path. and Bacteriol.*, 1911, 16, p. 247, 137.
10. *Ibid.*, 1911, 15, p. 323.
11. *Jour. Med. Research*, 1912, 27, p. 67.
12. *Jour. Infect. Dis.*, 1912, 10, p. 272.
13. *Ibid.*, 1913, 12, p. 144.

All were considered pneumococci that had well-developed capsules, that fermented inulin, that were green in blood-agar, and that were dissolved by bile. The results of the fermentation tests are given in the following tables.

Out of six strains of streptococcus hemolyticus only three were alike, and they were isolated from the same original culture.

TABLE 1

STREPTOCOCCUS HEMOLYTICUS

No.	Blood Agar	Source	Dextrin	Arabinose	Mannite	Salicin	Raffinose	Lactose	Saccharose	Inulin
3	Hemolysis	Blood (septicemia)	+	—	—	+	+	+	+	—
4	Hemolysis	Blood (septicemia)	+	—	+	+	+	+		
18*	Hemolysis	Tonsil (acute tonsillitis)	+	+	+	+	+	+	+	
19	Hemolysis									
20	Hemolysis	Tonsil (chronic arthritis)	+	±	—	—	±	+	+	—
27	Hemolysis									

* 18, 19, 20, different colonies from one culture.
+ = acid formed; — = neutral; ± = doubtful.

TABLE 2

PNEUMOCOCCUS

No.	Blood Agar	Source	Dextrin	Arabinose	Mannite	Salicin	Raffinose	Lactose	Saccharose	Inulin
11	Green	Sputum (Pneumonia)	+	—	—	+	+	+	+	+
23	Green	Cerebrospinal Fluid	+	+	+	+		+	+	+
25	Green	Sputum (Pneumonia)	—	—	+	+	—	+	+	+
32	Green	Blood Culture (Pneumonia)	+	—	—	—	+	+	+	+
35	Green	Pneumonia Cerebrospinal Fluid	+	+	—	—	+	+	+	+

+ = acid formed; — = neutral.

Of the five strains of pneumococci no two strains were alike.

Table 3 includes the streptococci that produced green in blood-agar, and which we call the streptococcus viridans. The green-producing cocci that peptonize milk are classed as the streptococcus zymogenes.[14]

14. *Jour. Exper. Med.*, 1899, 4, p. 521.

Table 3 shows the behavior of the streptococcus viridans on arabinose, dextrin, salicin, raffinose, mannite agar, unless otherwise indicated. For example, Strains 13, 14 and 28 fermented all five carbohydrates.

A study of these results, and a comparison of them with those of previous workers will lead one to conclude that there is a remarkable variation in the fermentative reactions of the streptococci.

TABLE 3

STREPTOCOCCUS VIRIDANS

No.	Source					
13 14 28	Blood culture (endocarditis) Tooth ..... Tonsil	+	+	+	+	+
7 12 15 16 17 26	M. rheumaticus B. ..... Blood culture (endocarditis) Tooth ..... Eye ..... Infected wound ..... Skin	+	+	+	+	Raffinose —
5 6 8 21	Urine ..... Anus ..... Tonsil ..... Blood culture (endocarditis)	+	+	+	+	Arabinose —
29	Tooth	+	+	+	+	Mannite —
31	Blood culture (endocarditis)	+	+	+	Raffinose —	Mannite —
34	Tooth	+	+	+	Salicin —	Mannite —
30	Tooth	+	+	Dextrin —	Raffinose —	Mannite —
10	Tooth	+	+	Arabinose —	Raffinose —	Mannite —
33	Tooth	+	+	Salicin —	Raffinose —	Mannite —
24	Joint	+	Arabinose —	Salicin —	Raffinose —	Mannite —
9	Tooth	+	Arabinose —	Dextrin —	Raffinose —	Mannite —
1	Blood culture (endocarditis)	+	Arabinose —	Dextrin —	Raffinose —	Mannite —
2	Blood culture (endocarditis)	+	Arabinose —	Dextrin —	Raffinose —	Mannite —
22	Cerebrospinal fluid	—	Arabinose —	Dextrin	Raffinose —	Mannite —

The problem now seems to be to determine, if possible, the cause of these great variations.

One colony of Strain 12, obtained from the blood in a case of infectious endocarditis, was plated and ten colonies were transplanted to agar tubes.

The ten colonies were transplanted to sugar-free lactose broth and after determining that the growth was pure, phenolphthalein was

TABLE 4

ACID REACTION OF TEN COLONIES FROM STRAIN 12

No. of Colonies	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
1	N	+	++	+	+	+	N	++	++	+++	++	++	++	+++	+++	+++	+++	+++
2	N	++	++	++	+	+	N	++	++	++	++	++	+++	++	+++	+++	+++	+++
3	++	+	++	++	+	+	N	++	++	++	+++	+++	+++	+++	+++	++	+++	+++
4	N	++	++	+++	++	+	N	+++	++	++	+++	++	+++	+	+++	+++	+++	+++
5	++		++	+++	+	+	N	++	++	++	+++	++	+++	+++	+++	+++	+++	+++
6	++		++	++	+	+	N	++	++	++	++	++	+++	++	++	+++	+++	+++
7	++		+++	++	+	+	+	++	+++	++	++	++	+	+	N	+	+	++
8	++		++	+	+	+	N	+++	++	++	++	++	+	+	++	++	++	+
9	N	++	++	N	+++	+++	N	++	++	++	+++	++	+++	+++	+++	+++	+++	+++
10	++		++	+	+	+	+	++	++	+++	++	++	+	+	++	++	+++	+++

N = neutral; + = faintly acid; ++ = distinctly acid; +++ = very acid.
The figures I-XVIII refer to the different transplants.

added and the broth was titrated, but no increase in acidity was detected. Later, these colonies would not grow in the sugar-free broth containing lactose nor in the same added to agar.

The ten colonies were transplanted to dextrin sugar-free agar, and the results are in Table 4.

Even in the first trial there was variation of the colonies, since in six of the tubes the reaction was acid, while in the remainder no fermentation took place.

On the third trial Colony 7 produced such a large amount of acid that it suggested contamination, but on plating it out, it was found to be pure.

On the fifth and sixth trials the dextrin was filtered through a Berkefeld filter and not heated, but there was still variation in the amount of acidity.

That the variation was not due to lack of uniformity in the titration or in the dextrin, is proved by the great discrepancy between the eighth and ninth trials, which were made on the same lot of agar, and between the tenth, eleventh and twelfth trials, which likewise were grown on the same lot of agar.

With successive inoculations on dextrin it was found that, as a rule, the power to produce acid was increased. The exceptions to this rule, Colonies 7 and 8, which formed very little acid in the last trials, died out soon after the trials were made.

In the thirteenth trial, Colonies 1 and 10, only, were in ordinary glass and there was very little acidity, but there was likewise very little acidity in 7 and 8, grown in the quartz tubes.

In the fourteenth trial Colonies 1 and 9, only, were in ordinary glass and there was just as much acidity as the most produced in quartz glass, also there were great variations in the amount of acidity produced by the different colonies in quartz tubes. In Colony 10, which was very faintly acid, the growth was scanty. It might seem that the amount of growth is an important factor, but there were instances in which the growth was alike with great variations in acidity.

In the fifteenth trial, Colony 7, in a quartz tube, did not produce any acid at all, and Colony 10 produced only a slight amount.

In the sixteenth trial, Colonies 3, 7 and 8, grown in the quartz tubes, produced less acid than the other colonies.

Colony 1 was grown in both ordinary and in quartz tubes, and both were very acid. The agar was decolorized near the bottom in the ordinary glass tube; but in this tube there was a greater distance from the bottom of the tube to the surface of the agar. In other instances, however, the depth of the agar did not influence the decolorization.

In one case where the same colony was grown in both ordinary and in quartz glass there was less acid produced in the ordinary glass.

In the eighteenth trial the cultures were grown under anaerobic conditions, and Colonies 7 and 8 produced less acid than the others. After this trial Colonies 7 and 8 died.

It was noted in the sixteenth trial that one colony grown in the quartz tube was not so acid as the same colony grown in the ordinary glass and that the latter had water of condensation present. In order to learn if the water affected the result, the same colony was simultaneously inoculated on tubes of media from which the water of condensation had evaporated, and on some of these tubes that had distilled water added to them after which they were sealed. In the latter case more acid was produced. The water may have aided the dissemination of the acid through the media.

The ten colonies of Strain 12 were planted in nutrose water medium plus 1 per cent. of dextrin. The medium had been previously incubated, and found sterile. All the colonies grew well in this medium. At the first trial there were marked differences varying from neutral, in two colonies, to marked acidity. This same medium was placed in quartz test tubes and the two colonies mentioned above remained neutral.

In the following experiments the ten colonies were obtained from one colony.

Ten colonies of Strain 30 were planted twice on raffinose agar without any variations appearing, since none of the colonies fermented this substance.

Ten colonies of Strain 28 were planted twice on raffinose agar and on the second trial there was a slight variation. Some did not ferment at all while others produced a slight acidity.

Ten colonies of Strain 36 were planted on raffinose agar. The medium was freshly made and the water of condensation was present. There was a good growth in every tube. At the first trial all were neutral except Colonies 8 and 10, which were strongly acid. At the next trial, on the same lot of media, similar results were obtained, but Colony 8 was not so distinctly acid. The ten colonies were then

placed on mannite agar and all were neutral except 8 and 10, which were moderately acid.

Ten colonies of Strain 37 were planted on raffinose agar and at the very first trial there was marked variation which ranged from neutral to marked acidity. Colonies 1, 2, 6, 9 and 10 were neutral 3 and 8 were faintly acid, and the remainder were markedly acid. When these ten were planted on mannite they all produced a large amount of acid. Ten colonies derived from one colony of Strain 13 were placed on arabinose for four successive times. On the first trial two did not ferment and the remainder were doubtful. On the second trial Colony 10 produced more acid than the remainder. On the third all but Colony 4 produced acid. On the fourth trial all the colonies fermented the sugar extensively.

In the experiments with the micrococcus rheumaticus, ten colonies were obtained as follows: a streak culture was made on agar in a petri dish and one colony was fished off and a streak culture made on each of two petri dishes, and the ten colonies were then fished from the second dish.

The first trial was made on raffinose in sugar-free litmus agar tubes that were old. There was no water of condensation present. None produced acid.

The second trial was made on fresh agar containing raffinose. All were neutral except Colony 5, which changed the entire amount of agar to a distinct red. Similar results were obtained on the third and fourth trials.

Colony 5 was streaked on agar in a petri dish and the five colonies fished off distinctly acidified raffinose, while five colonies from Tube 4, which was neutral, did not acidify raffinose at all. The ten original colonies were planted on arabinose and all (except Colony 5, which remained neutral) produced a large amount of acid.

These two distinct colonies were plated in blood-agar and found to be green. The growth was also similar in broth and in dextrin litmus agar. In mannite litmus agar, one produced more acidity than the other.

Therefore it seems that there were two distinct strains in this culture, or else we have here another instance of variation.

The result of experiments with the micrococcus rheumaticus are given in Figure 1.

I compared my results with those obtained by Rosenow,[15] using the same culture of the micrococcus rheumaticus, and found that we disagreed in our results in the cases of three carbohydrates: raffinose, inulin and mannite.

Miss Jean Broadhurst (using the titration method) tried out three of my strains and her results agreed with mine.

The behavior of the colonies of Strain 12 on lactose is interesting. When first isolated they grew luxuriantly in the lactose sugar-free broth, but after a number of transplants on dextrin, they were placed on lactose agar made of sugar-free broth. Only half of the colonies grew, and the fermentation of lactose was irregular. This experiment was repeated several times with similar results. Recently the colonies were planted in sugar-free broth containing lactose, but none grew. On repetition, the same result was reached. Other strep-

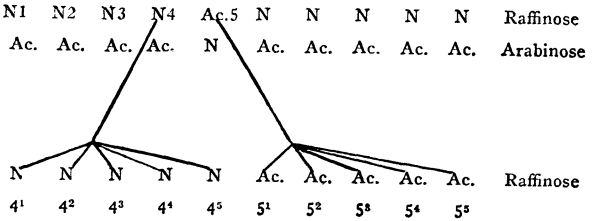

Fig. 1.—Result of Experiments with the micrococcus rheumaticus.

tococci grew in this broth luxuriantly. Thinking that the colonies could be brought to grow on the sugar-free agar they were transplanted to litmus milk. There was abundant growth. They were then transplanted to broth, not sugar-free, where there was good growth. They were then put back on the lactose agar made of sugar-free broth but there was no growth whatever. It seems that as the colonies began to die out their power to grow in a sugar which they did not ferment became diminished.

Six colonies fished from the original plate, made at the time of the removal of a piece of the tumor from the nose in a case of rhinoscleroma, gave variable results. Colonies 1 and 2 fermented lactose, while the remainder did not. Colonies 1, 2 and 3 fermented saccharose, while the remainder did not. This cannot be due to scanty growth, since the growing powers of this organism are well known.

15. *Jour. Infect. Dis.*, 1910, 7, p. 413.

In order to learn whether long cultivation of organisms on artificial media changed their fermentation power, two strains of *Bacillus mucosus,* which had been kept alive for nearly three years, were tried out on the same carbohydrates. In one strain dulcite had not been fermented, but it did produce acid on the second trial. With the other strain the power to ferment dextrin was lost. A strain of *Bacillus rhinoscleromatis,* which had been grown on artificial media for three years, had lost the power to ferment dextrin.

CONCLUSIONS

The variability in the results of the action of bacteria on certain chemical substances may be due to:

The use of media that have become dry and lost the water of condensation.

Faulty preparation of media: not exhausting the meat sugar from the broth, or overheating the carbohydrate.

The variability in the growing powers of different strains of streptococci and pneumococci.

Length of time grown on artificial media.

The complexity of the substances used and the difficulty of obtaining absolutely pure chemicals: dextrin, arabinose, etc. Different investigators have not used the same samples of carbohydrates.

The human element which enters into the titration of the media, and it is probable that the reaction varies with different workers.

A small amount of alkali from glass tubes which would be sufficient to change a faint acid reaction to a neutral.

The exceptional variability of the group of bacteria. It seems that some of these experiments prove this to be true.

Some of the disadvantages are obviated by the use of a liquid medium instead of agar. But if liquid media are used the results are still modified by the alkali in the glass, overheating, complexity and variability of the chemicals, variability of the growth, and the human element in the titration.